STAIN TECHNOLOGY

VOLUME VIII OCTOBER, 1933 NUMBER 4

METHODS FOR THE STANDARDIZATION OF BIOLOGICAL STAINS[1]

PART III

A. R. PETERSON,[2] H. J. CONN, and C. G. MELIN[3]

Contribution No. 229 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Dept. of Agric., Washington, D. C.

CERTAIN NITRO AND AZO DYES

ABSTRACT.—**In this paper are given the methods for determining the suitability of certain dyes of the nitro and azo groups for certification by the Commission on Standardization of Biological Stains. These methods have been developed by the Commission in cooperation with the Color and Farm Waste Division, Bureau of Chemistry and Soils, U. S. Department of Agriculture. The dyes for which the methods are given in the present paper are: Martius yellow, orange G, Janus green B, methyl orange, orange II, Sudan III, Sudan IV, Bismarck brown Y, Bismarck brown R, Congo red, and Erie garnet B. For each of these dyes, methods are discussed under the following headings: (1) identification or qualitative examination; (2) quantitative analysis; and (3) biological tests.**

Methods are given in this paper for the standardization of stains of the nitro and azo groups.[4] In order to eliminate considerable repetition in the discussion of the individual methods, many of the details which are the same for all of the dyes in this group will be outlined below.

For identification the dye is dissolved in a solvent in which, on dilution, there is no change in the location of its spectral band, thus making it unnecessary to prepare solutions of known concentration. Its extinction coefficient (E) is measured at two wave lengths,[5] one on

[1]The previous papers of this series have appeared in Stain Techn., **8**, 87, 95. 1933.

[2]Research Fellow supported by the Commission on Standardization of Biological Stains.

[3]Assistant Scientific Aide, Color and Farm Waste Division, Bureau of Chemistry and Soils, Washington, D. C.

[4]A general discussion of the methods of analysis and identification is given in the first paper of this series, Stain Techn., **8**, 87. 1933.

[5]Wave lengths are expressed in millimicrons, ($m\mu$).

each side of its maximum; that is, one on each slope of its absorption curve.[6]

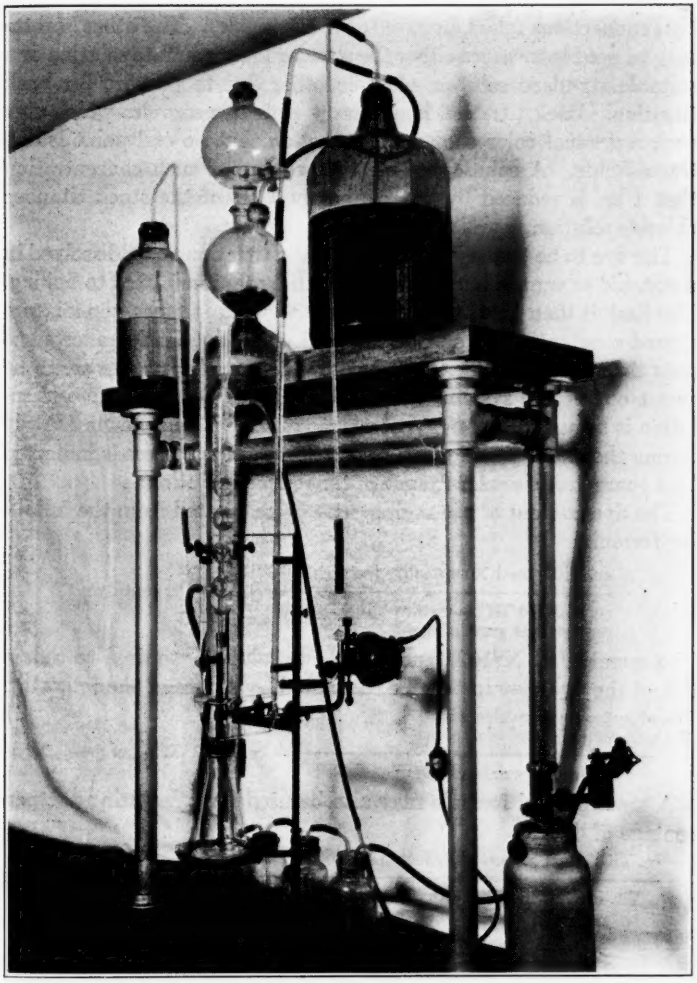
The formula to be given for each stain is that of the anhydrous dye as it is most commonly found on the market. The anhydrous dye is arbitrarily chosen to eliminate disagreement due to water of hydration. It is also necessary to warn the analyst that the salt-forming radical associated with the dye may not be the one given, basic dyes in particular appearing as salts of different acids and acid dyes as various metallic salts. Changing the acid radical with which a basic dye is combined does not affect the absorption ratio, but distinct differences in the ratios are obtained from the different metallic salts of an acid dye. Before the dye content of the sample to be examined can be accurately calculated, the exact nature of the dye must be known.

The determination of actual dye in stain samples of these groups by reduction with titanous chloride has been found satisfactory when the technic followed is varied to conform with the optimum conditions for the individual dyes under consideration. The titanous chloride solution employed in these titrations is approximately one-tenth normal. A solution of this concentration is prepared by diluting 75 cc. of 20% commercial titanous chloride solution and 75 cc. of concentrated hydrochloric acid (sp. gr. 1.18) to 1000 cc. with distilled water. The titanous chloride solution is readily oxidized by atmospheric oxygen and is therefore kept under an atmosphere of hydrogen, supplied from a Kipp generator which is connected to the bottle containing the stock solution.

The solution is standardized by means of one-tenth normal ferric sulfate solution. To prepare the ferric sulfate solution, 5.5924 g. of ingot iron (Bureau of Standards sample No. 55, which contains 99.85% iron) are dissolved in 30 cc. of concentrated sulfuric acid (sp. gr. 1.84) and diluted to 400 cc. with distilled water. A solution of pure potassium permanganate (3.16 g. dissolved in 200 cc. of water) is added slowly with stirring until a slight reddish color appears. The mixture is then diluted to one liter. Twenty cubic centimeters of this solution are titrated in an atmosphere of carbon dioxide with the titanous chloride solution prepared as above. Near the end point 5 g. of ammonium sulfocyanate are added and the addition of titanous chloride continued until a colorless end point is reached.

The titration is carried out in a wide-mouth 500 cc. Erlenmeyer flask, with a stationary rubber stopper fitted with two burettes, a

[6] In the case of a dye whose maximum absorption is below 500 mμ it has been customary to make both readings on one slope of the curve, as it is difficult to obtain accurate readings below that wave length. In many cases the maxima of these yellow dyes lie outside the visible spectrum.



APPARATUS FOR THE TITRATION OF DYES WITH TITANOUS CHLORIDE

In use at the Color and Farm Waste Division of the Bureau of Chemistry and Soils, U. S. Department of Agriculture.

carbon dioxide inlet, a bulb condenser and a mechanical stirrer. One burette is connected to the titanous chloride solution by *air tight* connections (glass, in so far as it is possible). The other burette may be used to measure either the standard ferric sulfate solution or a suitable standard solution of some other dye to be used for back titration. Back titration is necessary with dye samples which give such a gradual color change on reduction that no end point is distinguishable. A solution of methylene blue of such concentration that 1 cc. is reduced by approximately 1 cc. of the stock titanous chloride solution, is well adapted to this purpose.

The dye to be tested is weighed into the titration flask, dissolved in a suitable solvent, a buffer added, and the solution heated to boiling. The flask is then fitted tightly to the stationary stopper, the mixture stirred constantly and a strong current of carbon dioxide allowed to pass thru.[7] After sufficient time has elapsed to allow the air to be swept out of the system, the addition of the titanous chloride solution is begun. The stream of carbon dioxide is kept flowing slowly during the entire titration. The end point in most cases is indicated by a characteristic color change of the reduced solution.

The dye content of the sample may be calculated from the following formula:

$$\frac{\text{cc. } TiCl_3 \text{ used} \times \text{normality factor} \times 10 \times 100}{\left.\begin{array}{l}\text{cc. of N/10 } TiCl_3 \\ \text{required per gram of dye}\end{array}\right\} \times \text{weight of sample}} = \text{Per cent dye}$$

The quantity of N/10 titanous chloride solution required to reduce 1 g. of the dye may in turn be calculated by means of the following:

$$\frac{\left.\begin{array}{l}\text{No. of hydrogen equivalents for} \\ \text{the reduction of 1 mol of dye}\end{array}\right\} \times 10 \times 1000}{\text{Molecular weight of the dye}} = \text{cc. N/10 } TiCl_3 \text{ per gram of dye}$$

A more general formula may also be used for calculating the percentage of dye:

$$\frac{\text{cc. } TiCl_3 \text{ used} \times \text{normality factor} \times \text{mol. wt. of dye} \times 100}{\text{Wt. of sample} \times \text{No. of hydrogen equivalents} \times 1000} = \text{Per cent dye}$$

MARTIUS YELLOW, C. I. No. 9[8]

Identification: Martius yellow is the monosodium or calcium salt of 2:4-dinitro-1-naphthol, $C_{10}H_5N_2O_5Na$ or $2(C_{10}H_5N_2O_5)Ca$. The absorption ratio of the sodium

[7] CAUTION: The carbon dioxide must be passed thru a series of wash bottles to remove all traces of air or oxygen from the gas. A strong solution of $TiCl_3$ has been found suitable for this purpose. All connections must be made air tight, as small leakages here cause appreciable error in the determination of dye content.

[8] This abbreviation indicates the number under which the dye is listed in the "Colour Index", published by the Society of Dyers and Colourists, Bradford, Yorkshire. 1924.

salt, (E at 500 mμ/E at 530 mμ), is approximately 17.8. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water. Under the same conditions, the value of the ratio for the calcium salt of the dye is 26.5. A marked distinction is also observed in the solubilities of the two salts in water, the sodium salt being soluble to the extent of 4.5 parts in 100 parts of water at 26° C., whereas only 0.05 parts of the calcium salt are soluble in 100 parts of water at the same temperature.

Method of Analysis: Dissolve not more than 0.1 g. of dye in 150 cc. of distilled water, add 10 g. of sodium acid tartrate and titrate with N/10 $TiCl_3$. Disappearance of the yellow color indicates the end point. The dye reduces slowly but the reaction is hastened noticeably if the solution is kept hot during the titration. The following data are used in calculating the percentage of anhydrous dye in the original sample:

(1)	Molecular weight, $C_{10}H_5N_2O_5Na$	256.053
	Hydrogen equivalents per mol of dye	12.
	cc. of N/10 $TiCl_3$ per gram of dye	468.660
or (2)	Molecular weight, $2(C_{10}H_5N_2O_5)Ca$	506.188
	Hydrogen equivalents per mol of dye	24.
	cc. of N/10 $TiCl_3$ per gram of dye	474.140

Biological Tests: For martius yellow no very satisfactory biological tests have yet been devised. It is rarely used as a biological stain, the most common procedure being in the Pianese IIIb stain, in which it is mixed with malachite green and acid fuchsin. The few samples of martius yellow that have been certified have been tested by this technic on plant tissue, using the procedure given at the bottom of page 41 of Biological Stains, 2nd edition.[9] This has not proved, however, to be a reliable test, inasmuch as in a good preparation stained by this technic the yellow is scarcely visible; and it is, accordingly, very difficult to tell a good from a poor sample.

ORANGE G, C. I. No. 27

Identification: Orange G is the disodium salt of benzene-azo-2-naphthol-6:8-disulfonic acid, $C_{16}H_{10}N_2S_2Na_2$. The absorption ratio of the dye, (E at 500 mμ/E at 530 mμ) is 5.24. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of dye in 100 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling, and titrate with a standard solution of N/10 $TiCl_3$. The end point is a sharp change from brownish-yellow to a pale yellowish-green The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	452.218
Hydrogen equivalents per mol of dye	4.
cc. of N/10 $TiCl_3$ per gram of dye	88.452

Biological Tests: Orange G is tested as a counterstain in histology and in cytology. For its histological use it is tested on animal tissue fixed in Bouin's or Zenker's solution and embedded in paraffin. A 0.5% solution in 95% alcohol is employed and applied for 15 seconds or more as a counterstain after Harris' or Heidenhain's hematoxylin. For

[9]Conn, H. J. Biological Stains. *2nd Ed.* Biological Stain Commission, Geneva, N. Y. 1929.

cytological work a saturated solution in oil of cloves is applied. Root tip material, fixed in Nawashin's fluid, is stained in a 1% aqueous solution of crystal violet, rinsed in water and transmitted to equal parts of 1% solution of iodine in 80% alcohol and 1% solution of potassium iodide in 80% alcohol for 15 to 60 seconds. The sections are next dehydrated in absolute alcohol for 4 to 5 seconds and differentiated in the orange G in clove oil. They are then cleared in xylol and mounted. In both of these procedures a stain is required which gives a good contrast to the nuclear dye.

JANUS GREEN B,[10] C. I. No. 133

Identification: Janus green B is diethylsafranin-azo-dimethylanilin, $C_{30}H_{31}N_6Cl$. The absorption ratio of Janus green, (E at 635 mμ/E at 665 mμ) is 0.97. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of dye in 150 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling and titrate with N/10 $TiCl_3$. The dye solution undergoes several color changes on reduction, but the final end point, usually a clear reddish-brown, is easy to recognize. The following data are used to calculate the percentage of anhydrous dye in the original sample:

Molecular weight	486.753
Hydrogen equivalents per mol of dye	6.
cc. of N/10 $TiCl_3$ per gram of dye	117.670

Biological Tests: Janus green is tested, mixed with neutral red, for the supravital staining of blood. Saturated stock solutions of the two dyes are prepared by adding 125 mg. of neutral red to 50 cc. of neutral absolute alcohol and 125 mg. of Janus green to 62.5 cc. of neutral absolute alcohol. Both of these solutions are stable. A dilute stock solution of neutral red is prepared by adding 50 drops of the saturated neutral red to 10 cc. of neutral absolute alcohol. This solution is also stable. In testing Janus green, 2 drops of the saturated solution of this dye are added to 3 cc. of the dilute solution of neutral red. This mixture is not stable and must be used promptly.

Slides are washed and dried carefully, after treatment in bichromate cleaning fluid, and are then flooded with the mixed stain, which is allowed to drain off. The slides are then dried quickly in front of a Bunsen flame. A drop of fresh blood is placed on a cover slip which is inverted on top of the slide having the film of dried stain. The edges of the cover slip must be sealed with vaseline of high melting point.

By this technic, with good samples of both dyes, the basophilic granules become brilliant scarlet, the eosinophilic granules yellow or light orange, while the neutrophilic granules are salmon color. It is essential that no staining of the nuclei should be obtained which, if it occurs, indicates that the cells are killed. All the areas that stain with neutral red take up the dye quickly, except in the case of monocytes, which require about 10 minutes. The mitochondria show up after about 15 minutes as small blue dots or rods. The cells should live in good condition for at least an hour.

[10]The formula given in the Colour Index for Janus green B has been found incorrect for all samples furnished in this country. The dye usually encountered as a biological stain has the formula given in this paper. However, German samples of the dye, altho similar, are not identical with the usual stain sample. The problem of determining the exact chemical nature of these German samples will be continued and reported in a later publication.

METHYL ORANGE, C. I. No. 142

Identification: Methyl orange is the sodium salt of p-sulfobenzene-azo-dimethylanilin, $C_{14}H_{14}N_3O_3SNa$. The absorption ratio of the dye, (E at 500 mμ/E at 530 mμ) is 0.97. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water, plus 10 drops of 6 N. HCl per 50 cc.

Method of Analysis: Dissolve 0.2 g. of dye to be tested in 150 cc. of distilled water, add 15 g. of sodium acid tartrate, heat to boiling and titrate with a standard solution of N/10 $TiCl_3$. The end point is sharp, giving a colorless solution. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	327.197
Hydrogen equivalents per mol of dye	4.
cc. of N/10 $TiCl_3$ required per gram of dye	122.250

Biological Tests: Methyl orange is tested in saturated alcoholic solution as a counterstain, applied for 1 or 2 minutes following Harris' or Heidenhain's hematoxylin, on paraffin sections of animal tissue fixed in Bouin's or Flemming's fluid. A good sample is one which shows a good contrast to the nuclear stain.

ORANGE II, C. I. No. 151

Identification: Orange II is the sodium salt of p-sulfobenzene-azo-2-naphthol, $C_{16}H_{11}N_2O_4SNa$. The absorption ratio of Orange II, (E at 500 mμ/E at 530 mμ) is 1.95. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of dye in 150 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling, and titrate with N/10 $TiCl_3$. Reduce to a practically colorless solution. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	350.165
Hydrogen equivalents per mol of dye	4.
cc. of N/10 $TiCl_3$ per gram of dye	114.230

Biological Tests: Orange II is tested by the same procedure as in the case of methyl orange.

SUDAN III, C. I. No. 248

Identification: Sudan III is benzene-azo-benzene-azo-2-naphthol, $C_{22}H_{16}N_4O$. The absorption ratio of the dye, (E at 500 mμ/E at 530 mμ) is 1.11. The solvent employed is 95% alcohol.

Method of Analysis: The low solubility of this dye interferes with its determination, and it is therefore sulfonated before titration. Weigh about 1 g. of dye into a 125 cc. beaker-flask, add 25 cc. of 23% fuming sulfuric acid, and heat on the steam bath for at least one hour. Allow the mixture to cool and pour into 500 cc. of distilled water in a one-liter volumetric flask. After cooling to room temperature, the solution is brought up to the mark.[11] Pipette a 100 cc. aliquot of the sulfonated solution into the titration flask, add 100 cc. of distilled water and 30 g. of sodium acid tartrate, heat to boiling, and titrate with N/10 $TiCl_3$. The color change near the end point is very gradual, going from orange to a lemon-yellow. The disappearance, however, of the last trace of

[11]The red solution obtained on the dilution of the blue sulfonation product should be clear. If the sulfonation has been incomplete, the solution is cloudy.

orange is easily detected. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	352.160
Hydrogen equivalents per mol of dye	8.
cc. of N/10 $TiCl_3$ per gram of dye	227.170

Biological Tests: Sudan III is tested as a fat stain. Frozen sections of formalin-fixed tissue containing fat, or portions of the mesentery of some animal, serve for material. A saturated solution in equal parts of 70% alcohol and pure acetone is employed. The procedure given at the bottom of page 50 of Biological Stains, 2nd edition, is followed, using some hematoxylin formula as a counterstain. If the fat globules show distinctly, the dye is regarded as satisfactory. Sudan III is not expected to give as intense a coloration as Sudan IV.

SUDAN IV, C. I. No. 258

Identification: Sudan IV is o-toluene-azo-o-toluene-azo-2-naphthol, $C_{24}H_{20}N_4O$. The absorption ratio of the dye, (E at 500 mμ/E at 530 mμ) is 0.97. The solvent employed is 95% alcohol.

Method of Analysis: The low solubility of this dye interferes with its determination, and it is therefore sulfonated before titration, by the same procedure as that outlined for Sudan III. The percentage of dye in the original sample is calculated from the following data:

Molecular weight	380.192
Hydrogen equivalents per mol of dye	8.
cc. of N/10 $TiCl_3$ per gram of dye	210.420

Biological Tests: Sudan IV is tested by the same procedure as in the case of Sudan III. A good sample should give a sharp reddish-orange color to the fat globules.

BISMARCK BROWN Y, C. I. No. 331

Identification: Bismarck brown Y is the dihydrochloride of benzene-m-diazo-bis-m-phenylenediamine, $C_{18}H_{20}N_8Cl_2$. The absorption ratio of this dye, (E at 500 mμ/E at 530 mμ) is approximately 1.88. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of the dye to be tested in 100 cc. of 95% alcohol and 100 cc. of distilled water, add about 10 g. of sodium acid tartrate, heat to boiling, and titrate with N/10 $TiCl_3$. The end point, which ranges from pale yellow to brownish-yellow, is not always sharp, and is best observed by adding the titanous chloride solution 4 drops at a time. The following data are used for calculating the percentage of anhydrous dye in the original sample:

Molecular weight	419.138
Hydrogen equivalents per mol of dye	8.
cc. of N/10 $TiCl_3$ per gram of dye	190.860

Biological Tests: Bismarck brown Y is tested in a saturated aqueous solution, for staining mucous in goblet cells of the intestine, or cartilage in the trachea or in embryonic material, fixed in one of the usual fixatives and embedded in paraffin. The sections are stained for about 5 to 10 minutes, rinsed in alcohol, and transferred to the 0.5% aqueous methyl green solution until they appear dark green, after which they are dehydrated, cleared and mounted. A good Bismarck brown should show light brown mucous and deep brown cartilage.

Bismarck brown is also tested in a solution of the same strength on plant sections (fixed in formalin-alcohol-acetic acid and embedded in paraffin) as a counterstain against Delafield's hematoxylin. Employed in this procedure, a good sample should give satisfactory differentiation against hematoxylin.

BISMARCK BROWN R, C. I. No. 332

Identification: Bismarck brown R is the dihydrochloride of toluene-2:4-disazo-bis-m-toluylenediamine, $C_{21}H_{26}N_8Cl_2$. The absorption ratio of the dye, (E at 500 mμ/E at 530 mμ) is approximately 1.58. The solvent employed is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of the dye to be examined in 100 cc. of 95% alcohol and 100 cc. of distilled water, add 10 g. of sodium acid tartrate, heat to boiling, and titrate with N/10 $TiCl_3$. The end point, which ranges from pale yellow to brownish-yellow, is not always sharp, and it is best observed by adding the titanous chloride solution 4 drops at a time. The following data are used for calculating the percentage of anhydrous dye in the original sample:

Molecular weight	461.186
Hydrogen equivalents per mol of dye	8.
cc. of N/10 $TiCl_3$ per gram of dye	173.460

Biological Tests: Bismarck brown R has not been put on the certification basis, so no definite biological tests have been devised for it. One sample, however, has been submitted for certification by mistake in place of Bismarck brown Y, and the differences were so slight that there is little question but that it could be used for the same purposes and tested by the same procedures.

CONGO RED, C. I. No. 370

Identification: Congo red is the disodium salt of diphenyl-disazo-bis-1-naphthylamine-4-sulfonic acid, $C_{32}H_{22}N_6O_6S_2Na_2$. The absorption ratio of the dye, (E at 500 mμ/E at 530 mμ) is 1.23. A mixture of equal volumes of 95% alcohol and distilled water is used as the solvent.

Method of Analysis: Dissolve 0.1 g. of the dye in 125 cc. of distilled water, add 30 cc. of 30% sodium tartrate solution, heat to boiling, and titrate with N/10 $TiCl_3$. Titrate rapidly until near the end point, then slowly. The end point is sharp, a practically colorless solution resulting. Data for the calculation of the percentage of anhydrous dye in the sample are as follows:

Molecular weight	696.346
Hydrogen equivalents per mol of dye	8.
cc. of N/10 $TiCl_3$ per gram of dye	114.880

Biological Tests: Congo red is tested on paraffin sections of animal tissue fixed in Bouin's fluid. The sections are stained 5 minutes in Mayer's hemalum, dipped in tap water once or twice, and transferred directly into 0.5% aqueous solution of Congo red and left therein for 1 minute. They are then run up thru the alcohols, cleared and mounted. The criteria by which the sample is judged are as follows: Congo red should give a bright cytoplasmic stain with a certain amount of differentiation from orange to reddish. Erythrocytes, for instance, should be light orange and the spindle fibers in mitotic figures should be deep orange to light red.

ERIE GARNET B, C. I. No. 375

Identification: Erie garnet B is the disodium salt of diphenyl-disazo-4-sulfo-1-naphthylamine-1-naphthol-4-sulfonic acid, $C_{32}H_{21}N_5O_7S_2Na_2$. The absorption ratio, (E at 500 mμ/E at 530 mμ) is 0.94. The solvent used is a mixture of equal volumes of 95% alcohol and distilled water.

Method of Analysis: Dissolve 0.2 g. of the sample to be tested in 150 cc. of distilled water and 50 cc. of 95% alcohol, add 25 cc. of 30% sodium tartrate solution, heat to boiling and titrate with N/10 $TiCl_3$. As the end point with this dye is very poor, it is advisable to add a slight excess of $TiCl_3$ and back titrate with a standard solution of methylene blue. The following data are used in calculating the percentage of anhydrous dye in the original sample:

Molecular weight	697.320
Hydrogen equivalents per mol of dye	8.
cc. of N/10 $TiCl_3$ per gram of dye	114.720

Biological Tests: No definite biological tests have yet been worked out for Erie garnet B. The dye has seldom been used by biologists but was recently called for by Geschickter[12] in certain procedures for staining tumor tissue. It was in connection with this technic that the dye was put on the certification basis. Biological procedures for testing it will be worked out when there is more call for the stain among biologists.

[12]Geschickter, C. F. Fresh tissue diagnosis in the operating room. Stain Techn., 5, 81. 1930.

THE NATURE OF THE REACTION OF THE COLON ORGANISM ON ENDO'S MEDIUM

LUBOW A. MARGOLENA, *Commission on the Standardization of Biological Stains*, and P. ARNE HANSEN, *New York Experiment Station, Geneva, N. Y.*

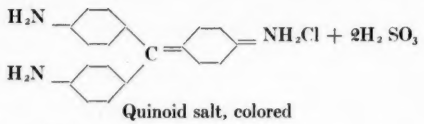
ABSTRACT.—***Bacterium coli*** **was grown in a medium of a composition similar to Endo's medium with the exception that the sulfite, fuchsin and agar were left out. When the fuchsin-sulfite mixture was added after 24, 36 and 48 hours, or after 3 and 10 days of incubation at 37° C., no reaction appeared. The substance that causes the reaction is only formed when the bacteria are grown in the presence of sulfite. When picric acid and ether are added to the red compound produced by the colon organism in Endo's medium and the mixture shaken, the color remains in the watery layer and thus the dye color that appears in the medium is not restored fuchsin, but a new substance. The work confirms Neuberg's and Nord's theory that the Endo medium acts as a trapping agent for the intermediate product, acetaldehyde, which causes the Endo reaction.**

Among the stains which the Commission on Standardization of Biological Stains has to certify, basic fuchsin offers more problems than many others, the reason being a wide application in a variety of fields. Its use in the Endo medium, especially, adds to the difficulties in certifying this dye because the factors that determine its suitability for this particular purpose are not well known. The first step in studying Endo's reaction naturally seems to decide upon the nature of the chemical reaction involved. There is still a great deal of uncertainty present about this point in many of the current text books on bacteriology, some favoring the view that the formation of lactic acid brings about the reaction, others pointing out the possible presence of aldehyde. (*Vide infra* p. 133).

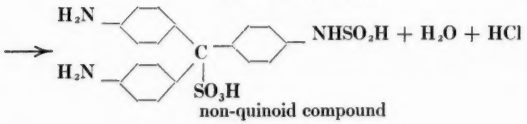
REVIEW OF LITERATURE

The main constituent of fuchsin is the chloride or acetate of the rosanilin or of pararosanilin salts which dissolve in water with the well-known fuchsia color. The dye reacts with sulfurous acid forming

(in the case of pararosanilin) a colorless compound by the following reaction:



Quinoid salt, colored

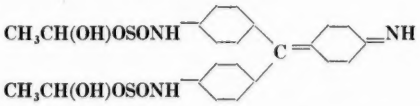


non-quinoid compound

When an aldehyde is added to the decolorized product, the sulfurous acid combines with the aldehyde and the color is "restored." It has long been known, however, that the regenerated dye is chemically somewhat different from the original dye and that the shade of color is slightly more purplish. Apparently the process must be more complicated than a mere removal of the sulfurous acid.

Thus Willstätter and Stoll (1918) made the observation that if picric acid is added to a watery fuchsin solution and the mixture then shaken up with ether, the picrate of fuchsin will completely go into the ether layer. But the product of condensation formed from the decolorized fuchsin + aldehyde behaves differently inasmuch as it does not pass into the ether at all. By means of Willstätter's test one is thus able to decide between the truly restored fuchsin on the one hand and a product of aldehyde condensation on the other. The two compounds also differ in their behavior toward acid; both fuchsin and aldehyde fuchsin are decolorized by strong acids, but fuchsin is the more easily attacked.

Wieland and Scheuing (1921) suggested the following formula for the aldehyde compound of pararosanilin:



Endo's original medium (1904) had the following composition: A solution consisting of meat infusion from 500 g. beef in 1 liter of water, 10 g. peptone, 5 g. NaCl, and 30 g. agar is prepared and neutralized, then 10 cc. of a 10% solution of soda, 10 g. of lactose, 5 cc. of alcoholic fuchsin and 25 cc. of 10% sodium sulfite added. He thought that the acid formed from lactose was the cause of the characteristic reaction, and this theory has often been repeated in the literature, altho acid sulfite is known to be a good decolorizer for fuchsin.

In 1916 Kendall writes in favor of the theory holding acid formation responsible for the reaction: "Those bacteria which ferment lactose—as *Bacillus coli*—form lactic acid which restores the color of the medium in the immediate neighborhood of the colony; the colony is therefore colored red. Some aldehydes also restore the color, but it is not very probable that aldehyde production is commonly observed among lactose-fermenting organisms. Non-lactose fermenting bacteria grow as colorless colonies."

Lehmann and Neumann (1931) explain that, "Traces of acids allow the red color to reappear" and further "only acid forming organisms color the colorless Endo medium red by the regeneration of the fuchsin."

Harding and Ostenberg (1912) decided that acetaldehyde was responsible for the Endo reaction. They write, "The aldehyde, as fast as produced, combines with the reagent and is fixed, thus being prevented from oxidizing to acid." Their view-point has a certain similarity with the theory of Neuberg and Nord (1919) discussed below, but the work was done, however, before the full significance of acetaldehyde as an intermediate product in the fermentation of the colon organism was realized. Many others have also claimed that acids could not cause the reaction and as an example we shall quote Zinsser (1929): "Although Endo described his medium as dependent upon the formation of acid by the bacteria, this is not so. Acids give no coloration of the sulphite-fuchsin mixture. Indeed this mixture is used by the chemists under the name of Schiff's reagent as a test for aldehydes. Acids decolorize the red caused by aldehydes, and this accounts for the frequent late discoloration of the red colon colonies on prolonged cultivation." The present writers, however have not observed this phenomenon.

Describing tests devised for determining the suitability of fuchsins for Endo's procedure, Conn (1926) writes, "It depends upon the principle of adding a measured amount of sodium sulphite to a weak fuchsin solution, determining the amount needed to effect decolorization, and then noticing the amount of a dilute formalin solution necessary to restore the color. In practice this proves to give results nearly parallel with those obtained in actual use." The same author (Conn, 1929), states later, "This Endo reaction is very similar to, if not identical with, the behavior of basic fuchsin in Schiff's reagent for detection of aldehyde."

For this latter view, that aldehydes cause the reaction, there is at first a slight objection that will, however, disappear at close examination: namely, the final amount of acetaldehyde formed in the lactose

peptone broth is so infinitesimally small that it is not possible to detect it (Neuberg and Nord, 1919). But this objection can be eliminated if we consider that the Na_2SO_3 acts as a "trapping agent" for CH_3CHO, as well as a decolorizer for the fuchsin.

Neuberg and Nord (1919) successfully applied the Neuberg trapping procedure (Abfangmethode) to the fermentation of the colon organism. The trapping procedure consists in fixing a certain intermediate substance in the scale of fermentation products and thus letting such a substance accumulate in the liquid. For acetaldehyde, secondary sodium sulfite was used and the writers concluded that acetaldehyde is formed as an intermediate product in the fermentation of glucose by this organism.

An outline is given below of the well-known set of reactions involved:

$$C_6H_{12}O_6 \longrightarrow 2\ CH_3.CO.C(H)(OH)-OH$$

glucose methylglyoxalhydrate

Part of the methyl glyoxal hydrate is transformed into $CH_3CHOH.COOH$ lactic acid, and part is further fermented as follows:

$$CH_3CO\ C(H)(OH)-OH \longrightarrow CH_3CHO + HCOOH$$

$$2\ CH_3CHO + H_2O \longrightarrow CH_3\ COOH + CH_3CH_2OH$$

$$HCOOH \longrightarrow CO_2 + H_2$$

In all experiments with the colon organism, Neuberg and Nord found that acetaldehyde was accumulated without question when Na_2SO_3 was added, while none was found when it was omitted. Neuberg and Nord, furthermore, suggested that the Endo reaction might be explained on this basis; they did not describe any experiments with Endo's medium.

This interesting view-point on the Endo medium has not, so far as we know, been considered any further and has been overlooked in text books, etc. In the following experiments some observations are described, which confirm the view of Neuberg and Nord.

Experimental

The formula of Endo's medium used in the experiments was Levine's (1918) modification of Endo's original medium; i. e. *Bacto* peptone, 10 parts; lactose, 10 parts; dibasic potassium phosphate, 3.5 parts; agar, 15 parts; sodium sulfite, 2.5 parts; basic fuchsin, 0.5 parts. The reaction which various chemicals give on Endo plates of the

composition given was tested by the following procedure. Plates were poured with 20 to 25 cc. of the medium and the agar was allowed to solidify. After hardening, a small disc of agar (about 5 mm. in diameter) was removed by means of a suitable glass tube and the chemical under test was placed in the cavity. The solid chemicals were mostly used in saturated solution except when otherwise noted.

The following reactions were given by various chemicals with Endo's agar by the above procedure:

Formaldehyde, commercial, 40%. purplish red color with metallic sheen
Acetaldehyde, 10% aqueous.... purplish red color with metallic sheen
Benzaldehyde..... purplish red color with metalic sheen
Vanilin, saturated, alcoholic..... very faint reaction, lavender color
Glycine, aqueous..... no color
Aspartic acid, aqueous..... " "
Asparagine, aqueous..... " "
Methylamine, 33% aqueous..... " "
Dimethylamine, 33% aqueous... " "
Trimethylamine, 33% "..... " "
Anilin..... purplish red color with metalic sheen
o-Toluidin, sat. alcoholic..... very faint reaction, lavender color
p-Toluidin, " " ..... faint reaction, lavender color
Diphenylamine, sat. alcoholic ... " " " "
Acetic acid, 10%..... pink reaction
Lactic acid, 10%..... " "
HCl, N/10..... " "
Na OH, N/10..... no reaction

Apparently, certain substances besides aldehydes will give production in the Endo plates; e. g., aromatic monoamines. Acetic and lactic acids produce a faint pink color, but it is unlike the deep and distinct red given by the aldehydes. The fact that a small increase in acidity brings about a faint color of the Endo medium, altho a larger increase brings about complete decoloration, has added to the general confusion in the theory of the mechanism of the Endo medium.

Table 1. Reaction between 9 cc. of liquid Endo medium and 1 cc. of lactic acid:

Lactic acid and solutions	Reaction
Undiluted	None
10%	Faint pink, or none
1%	None, or extremely faint
0.1%	None

In order to gain a more complete picture of the reactions involved, an Endo medium without the agar was prepared, which we shall refer to here as liquid Endo medium. Table 1 shows the results obtained by adding various dilutions of lactic acid. It will be seen that color production did not occur with the undiluted nor with the most dilute (0.1%) acid.

These results were obtained with varying grades of lactic acid (Baker's analyzed, Merck blue label, Merck white label and a special lactic acid, purified by ourselves thru the preparation of zinc lactate) but the outcome was the same with all samples. The use of different grades of fuchsin seemed, however, to have an influence on the deepness of the color and sensitivity. In no case was there a pronounced deep color reaction obtained, and the dye always passed into the ether layer when the mixture to which picric acid had been added was shaken with ether. The faint reaction which lactic acid causes in Endo's medium is distinctly different from the typical Endo reaction, as will be shown later. It must be kept in mind that some care in working with lactic acid is necessary, for it may contain aldehydes when handled without precaution. Thus, a solution of lactic acid containing some sulfuric acid will, when heated, decompose in part according to this reaction (Erlenmeyer, 1868)

$$CH_3\,CHOH\,COOH \longrightarrow CH_3\,CHO + H\,COOH$$

sufficiently to give a strong aldehyde reaction with fuchsin sulfurous acid.

It was demonstrated by a simple experiment that the fuchsin-sulfite mixture does not function as a simple indicator, but plays a role as a trapping agent, also changing the whole picture of fermentation. *Bacterium coli*[1] was grown parallel in three different media: 1) in the complete Endo medium, except for the agar; 2) in the same medium, minus the fuchsin; 3) in the same, leaving out both fuchsin and sulfite. The cultures were incubated at 37° C. and samples were removed, respectively, at 24, 36, 48 hours, and 3 and 10 days after inoculation. The first medium became purplish red, usually after 24 to 36 hours. If picric acid is added to this mixture, which is then shaken with ether, the color remains in the aqueous layer, as in the case of a reaction with acetaldehyde. When decolorized fuchsin was added to medium No. 3 (without the fuchsin-sulfite mixture) no reaction took place. This is in perfect agreement with Neuberg and Nord, who found that no acetaldehyde could be detected when the fermentation was carried out in a normal way without the addition of

[1]A culture of this name, denoted No. 71, obtained originally from Dr. Levine of Iowa State College, was secured thru the courtesy of Prof. Pauline Stark, of Cornell Univ.

sodium sulfite. It further shows that acid production cannot be held responsible for the production of the typical reaction on the Endo medium, as plenty of acid was produced in the lactose broth used. Furthermore, the reaction cannot be caused by any other end product of the fermentation.

When the decolorized fuchsin was added to medium No. 2, no reaction appeared. In this case it should, however, be kept in mind that a double quantity of sulfite was present (the amount originally present in the medium, and the second amount added with the decolorized fuchsin) making the detection of the small concentration of aldehyde present too difficult.

To complete these tests quantities of all three media were steam distilled after 10 days of incubation. To 100 cc. of the medium, 20 cc. of a 25% solution of barium chloride and 10 g. of calcium carbonate were added, after which about 150 cc. of the distillate were collected.

In the case of No. 1 and 2 (i. e., in media containing sulfite) an aldehyde reaction was obtained in the distillate, while none was obtained in medium No. 3, in which no sulfite was present. This plainly indicates the production of aldehyde by the colon organism, altho it does not accumulate in the absence of sulfite.

The Endo medium is not as highly sensitive to acetaldehyde as Schiff's reagent which is used in organic analysis as an aldehyde reagent. According to Willstätter, Schiff's reagent is sensitive at 1:1,000,000. The sensitivity of the Endo medium was determined as follows: To 10 cc. of the liquid Endo medium, made from Difco fuchsin and from Grübler's fuchsin, respectively, 1 cc. of aldehyde, in various dilutions, was added. It was found that a 0.5% solution gave a distinct reaction, while a 0.1% solution of acetaldehyde failed to do so. This was confirmed also when using a dehydrated Difco Endo agar.

The slow appearance of a purple color (in about 10 days, or sometimes even less) when the Endo medium is left in a 37° C. incubator is a complication probably brought about by oxydation of the sulfite. The color produced behaves in the Willstätter test as restored fuchsin. As acetaldehyde is also an intermediate product in alcoholic fermentation, it might be predicted that a yeast culture on a medium similar to Endo medium, but with glucose instead of lactose, would produce a growth with a deep red color and a metallic sheen resembling the characteristic growth of the colon organism on the Endo medium. A culture of *Saccharomyces ellipsoideus* was inoculated upon plates of Endo's medium with glucose as the source of carbohydrate instead of lactose, incubated at 20° C. for 4 days, after

which period the growth, so far as color and sheen were concerned, corresponded exactly to the typical Endo reaction for the colon organism.

Conclusions

1. Considerable confusion in the understanding of Endo's reaction is caused by the fact that a faint coloration may be produced by weak acids and by oxidations, both causing a restoration of the fuchsin color.

2. The view-point of Neuberg and Nord that the cultivation on the Endo medium is a trapping procedure (Abfangverfahren) for acetaldehyde is confirmed.

3. The typical reaction of *Bacterium coli* is caused not by lactic acid, but by the intermediate product acetaldehyde which is fixed by sodium sulfite.

4. The color produced in the reaction is not restored fuchsin, which is shown by the fact that it cannot be shaken out with ether when picric acid has been added beforehand.

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A METHOD FOR STAINING CERTAIN BACTERIA AND ANTHEROZOIDS

W. N. Steil, *Department of Botany, Marquette University, Milwaukee, Wis.*

Abstract.—**A modification of Loeffler's method for staining the flagella of bacteria was employed in staining large forms of bacteria and antherozoids. The bacteria or the antherozoids are killed and fixed in a drop of water on a slide and set aside to dry, before the next step is undertaken. The slide is treated for a period of time, varying from about ten minutes to several hours, in a practically saturated solution of tannic acid. After the slide is thoroly rinsed in water, it is stained with either a single stain or a combination of stains. The slide is then dehydrated with absolute alcohol, cleared with clove oil, and completed in the usual manner.**

The body of the bacterium and that of the antherozoid are well differentiated and the cilia are distinctly brought out by means of the method herein described.

The technic is of especial value in staining the antherozoids of mosses, liverworts, and ferns.

A modification of Loeffler's method for staining the flagella of bacteria was employed by the writer in the preparation of slides of large spirilla and bacilli and antherozoids of Bryophytes and Pteridophytes including those of *Marchantia polymorpha*, *Sphaerocarpus Donnellii*, *Riccardia pinguis*, *Onoclea sensibilus*, and *Marsilia quadrifolia*.

A drop of water containing the bacteria is placed on the slide, or on the cover slip, as is usual in bacteriological technic, and allowed to dry with or without the application of heat. The bacteria in the drop of water may also be killed and fixed by exposing the drop for about two minutes to the fumes of a 2% osmic acid solution. The slide is then set aside to dry. It may be bleached for a few minutes in a full strength hydrogen peroxide solution before staining is attempted.

The antherozoids of *Marchantia* are obtained free from foreign matter, including the slime from the antheridium, by first washing the mature male heads which have been removed from the thallus, with a stream of water from the tap, and placing them into a stender dish containing a small amount of clean water. Usually after a short

interval, a large number of motile antherozoids may be obtained. The non-motile antherozoids and the foreign matter sink to the bottom of the dish, and hence, a drop of water taken from the surface will usually contain only motile antherozoids.

The male plants of *Sphaerocarpus* and *Riccardia* and the fern prothallia are first thoroly washed in water and then placed into the stender dish containing a small amount of water. The sporocarps of *Marsilia* are sterilized with the standard bichloride of mercury solution and washed with distilled water before they are cut open and placed in water to germinate. Altho the development of the male gametophyte of *Marsilia* is known to occur within about 12 hours at ordinary room temperature, it has been found advisable to take the foregoing precaution to check the development of bacteria during the germination of the spores.

The antherozoids in a drop of water are killed and fixed with the fumes of 2% osmic acid solution and set aside to dry. Before being stained, the slide is bleached in a hydrogen peroxide solution, but little improvement is observed in a slide treated in this manner.

The slides of the bacteria and the antherozoids are placed into a coplin jar containing a saturated solution of tannic acid for a period of 10 minutes to several hours. The longer exposure of the slides to the tannic acid gives the better results. The slides are then removed from the acid solution and thoroly washed in water before staining is attempted.

Good results are obtained with either single stains or a combination of stains.

A typical staining technic in common use and involving a single stain is presented below:

1. Stain in safranin 10 minutes to several hours.
2. Rinse slide in water.
3. Dehydrate with absolute alcohol.
4. Clear with clove oil.
5. Complete slide in the usual manner by adding balsam and placing a cover slip on the mount.

When the above schedule is followed, the body of the bacterium stains a brilliant red, and the cilia a slightly fainter red. Gentian violet, crystal violet, light green, and fast green also give very fine results.

A very good contrast is obtained between the body and the cilia of the bacteria when a double stain is used. Safranin in combination

with gentian violet, crystal violet, light green, fast green, or orange G produces unusual contrasts. One of the best combinations of stains was found to be safranin and fast green. The body of the bacterium becomes intensely stained with the safranin and the cilia equally so with the fast green. Since the fast green acts quickly, only a few minutes exposure to the stain is necessary.

Good results may also be obtained with the Flemming triple stain. It has been found, however, much more difficult to use any of the triple stains in common use.

It was not difficult to apply the schedule successfully to the preparation of slides of antherozoids.

Especially good results may be obtained with antherozoids when some of the double stains are used, such as safranin and fast green, safranin and gentian violet, and safranin and orange G. Double stains produce good contrasts, not only in the parts of the body of the antherozoid, but also between the body and the cilia. For example, the nucleus stains brilliant red with the safranin, and the cytoplasmic portions green with the fast green. The vesicle stains more intensely than the other cytoplasmic portions of the body. The cilia also stain a green color.

All of the dyes used seemed to give satisfactory results. The certified dyes used for the staining schedules were as follows: Fast green (NGf-1), light green (NL-5) and methyl orange (NM-4) obtained from the National Aniline & Chemical Company. The uncertified dyes were Coleman & Bell Company products, as follows: Light green, fast green FCF, safranin A, safranin Y, orange G, gentian violet (improved), and crystal violet.

The safranin, gentian violet and crystal violet were prepared by adding the dry stain to a mixture of 100 cc. of 95% alcohol and 100 cc. of a 3% anilin oil solution in distilled water. The stain may be a practically saturated solution. The light green and the fast green were saturated solutions in 95% alcohol. The orange G used was a saturated solution in distilled water.

The method for staining which has been described is useful in staining the flagella of only large forms of bacteria and the antherozoids of Bryophytes and Pteridophytes. The cilia of some antherozoids, often difficult to stain by some of the usual methods, can be readily brought out by the method. The good differentiation in the body, especially of the antherozoids of plants, makes the method a desirable one. The antherozoid slides obtained by the technic herein described are superior to any other made by the writer

using the methods in common use, including the method described by him[1] elsewhere.

Good results were also obtained in the preparation of motile forms of protozoa. By means of the method the flagella can be distinctly stained.

It is suggested that the method may be used for staining the cilia of zoospores of algae and fungi.

[1]Steil, W. N. Method for staining antherozoids of ferns. Bot. Gaz., **65**, 562–563 1918.

BLOCK STAINING OF NERVOUS TISSUE WITH SILVER

III. Pericellular end-bulbs or boutons

H. A. DAVENPORT, *Department of Anatomy,*[1] *Northwestern University Medical School, Chicago*

ABSTRACT.—**Fixation in 20 to 40% pyridin or in 10% chloral hydrate followed by 10 to 40% pyridin gave the most consistent staining of pericellular structures in the spinal cord of cat. Chloral hydrate perfusion and soaking followed by ammoniated alcohol (Hoff's application of Cajal's method) was uniformly successful only when pyrogallol instead of hydroquinone was used as a reducing agent. Perfusion of the animal with chloral hydrate gave a rather questionable degree of improvement over fixation by simple soaking. The difficulty in selecting a routine procedure as the "best" became apparent when no single experimental variation was outstandingly superior in all animals.**

Considerable stimulus to interest in the function of the end-bulbs about the ganglion cells in the gray matter of spinal cord has been given recently by Hoff.[2] His finding that these structures undergo degenerative changes following experimental lesions seems so important that an experimental study of the staining methods which are known to show end-bulbs is justified.

EXPERIMENTAL

Spinal cord from 9 cats, 1 kitten (2 months), and 2 rats was used. Three of the cats were perfused with 10% chloral hydrate thru the aorta as recommended by Hoff. The cords of the other six were dissected out and put into fixative within an hour after death of the animals. The rats and kitten were not perfused.

Since previous experiments[3] with block staining indicated that fixation and other procedures prior to silver impregnation had the major effects on the stain, the present series was simplified by using 1.5% silver nitrate thruout for impregnation and either 2% pyrogallol or 2% hydroquinone for reduction.

The number of individual blocks of tissue prepared by any single procedure varied from 1 to 7. When a particular procedure, such as

[1]Contribution No. 194.

[2]Hoff, E. C. Central nerve terminals in mammalian spinal cord and their examination by experimental degeneration. Proc. Roy. Soc., London, Ser. B. **111**, 175. 1932.

[3]Davenport, H. A. Block staining of nervous tissue with silver. I. Stain Techn., **5**, 139, 1930, and II. Stain Techn., **6**, 37. 1931.

fixation in 5% pyridin, was obviously unsatisfactory it was abandoned after a single trial. More satisfactory ones, as fixation in 20% pyridin, were repeated with nearly all animals used. Procedures given in the following outline represent the total number of variations tried:

A. Fixation.
   1. 10% chloral hydrate:
      a. Perfusion only and perfusion plus soaking 24 hours.
      b. Soaking 3 hours, 16 hours, 1 day, and 3 days.
   2. 10% trichloracetic acid, 1 day.
   3. Pyridin: 5, 10, 20, 40, 60%, and pure.
   4. Ammoniated alcohol, 2 days.

B. Extraction or secondary fixation.
   1. Pyridin: 10, 20, 40%, and pure.
   2. Ammoniated alcohol, 1 day.

C. Washing (with water).
   1. Preceding silvering, none, 0.5 and 1 hour, 2 hours, and 2.5 days.
   2. After silvering, none, 0.5 hour, and 5 hours.

D. Silvering.
   1. 3 days and 7 days at 37° C.

E. Reduction.
   1. 2% Pyrogallol, 0.5 hour, 5 hours, and 24 hours.
   2. 2% Hydroquinone, 24 hours.

OBSERVATIONS

A. *Fixation.*

1. Perfusion by 10% chloral hydrate, altho as good stains of end-bulbs were obtained without perfusion as with it, seems to be a desirable procedure. The solution does not coagulate blood and when introduced into the aorta of the living, anesthetized animal is pumped thru the vascular system for 1 to 3 minutes before the heart stops. No choice was seen between preparations made by removing the cord immediately after perfusion and putting it into diluted pyridin or ammoniated alcohol and those made after 3, 16, or 24 hours soaking in chloral hydrate solution followed by the secondary fixative. The preparations soaked 3 days showed more shrinkage of the ganglion cells.

2. Trichloracetic acid fixation gave 2 good preparations out of 5 trials. A tendency toward overstaining of cells and production of a fine granular precipitate of silver rules it out as a desirable procedure.

3. Pyridin alone or following either chloral hydrate or trichlor-

acetic acid gave the most satisfactory displays of end-bulbs. Vacuolation of the gray matter was the chief fault when used alone, especially in 5 and 10% concentrations. In higher concentrations there was more tendency toward overstaining of cells. The concentration appeared to be uncritical but the practical optimum was 20 to 40%. Some end-bulbs always stained in all concentrations tried.

4. Ammoniated alcohol as a primary fixative gave inferior preparations.

B. *Extraction or secondary fixation.*

During soaking with 10% chloral hydrate or in 10 and 20% pyridin the myelin swelled and was extruded at the ends of the blocks. In either alcohol 40% pyridin, or pure pyridin this did not occur. No direct relationship between the degree of removal of the myelin and stainability of end-bulbs was noted excepting that the stain did not penetrate well nor were the end-bulbs abundant after chloral hydrate without subsequent extraction with either pyridin or alcohol. Pyridin solutions appeared to be definitely superior to ammoniated alcohol for showing the largest number of end-bulbs altho the impossibility of standardizing the occurrence of end-bulbs precludes absolute judgment on the relative merits of the two reagents.

C. *Washing.*

1. Washing preceding silvering was relatively uncritical after the more dilute pyridin solutions. One successful preparation was transferred directly from 20% pyridin to the silver bath with only rinsing for a few seconds. Washing more than 2 hours was shown to be unnecessary and preparations washed 2.5 days were inferior to those washed 1 and 2 hours. Brief washing (0.5 hour) and no washing were the only variations tried after ammoniated alcohol. The washing reduced the amount of precipitate formed in the periphery of the block but no effect on the staining of the center appeared.

2. Washing after silvering was definitely a critical procedure (confirming Hoff). Unwashed preparations had an over-stained periphery and thin blocks had no usable center. Preparations washed 0.5 hour often showed a superficial as well as a central zone in which the end-bulbs were differentiated. Blocks washed 5 hours were pale and the end-bulbs unstained.

D. *Silvering.* Any deepening of the stain after 3 days impregnation preceeded very slowly as judged by direct comparisons of blocks impregnated 3 days with those impregnated 7 days, hence the time was uncritical within the limits studied.

E. *Reduction.* Pyrogallol was much superior to hydroquinone as a reducing agent in this series as it has been for other variations of silver staining tried in this laboratory. When successful, hydroquinone gave brilliantly differentiated preparations but its reduction was usually spotty in character and the silver deposited was coarse grained. Five hour's reduction time with 2% pyrogallol gave the best stains. Blocks reduced only 0.5 hour showed incomplete reduction about 0.5 mm. inside of the block. Reduction for 24 hours added nothing and perhaps even detracted from the chances for a good stain.

Discussion of Results

Rat. Very few end-bulbs were seen in the spinal cord of rat. This material is either unsuitable for study or radically different methods must be used to demonstrate them if they occur in abundance.

Kitten. The staining reactions appeared to be similar to those of the adult cat but the number of end-bulbs shown were probably less than 1% as numerous. Chloral hydrate fixation for 3 hours followed by either ammoniated alcohol or 10% pyridin or fixation in 10% and 20% pyridin alone showed bulbs as numerous as 12 to 15 on a few cells in 12 μ sections.

Cat. The best preparation, judged by the abundance and good differentiation of bulbs was obtained after chloral hydrate perfusion under ether followed by immediate removal of the cord. Subsequent treatment was: Pyridin 10%, 2 days; washed 0.5 hr.; silvered 7 days; washed 30 min.; and reduced with 2% pyrogallol 5 hrs. Other specimens stained in a similar manner indicated that such a procedure was a favorable one.

Two other preparations of almost equal excellence were obtained from animals which had not been perfused. One of these was fixed in 20% pyridin for 3 days, washed 2 hrs., and treated subsequently the same as the preceding. The other was fixed 1 day in 10% trichloracetic acid, washed 2 days; then 20% pyridin 2 days; washed 1 day, and treated subsequently similar to the other two.

There was no specimen which did not show some end-bulbs somewhere in the block and their general occurrence on a single cell varied from a few to hundreds. Just how much this variation was due to natural presence of the pericellular terminals and how much due to staining was not determinable. Both factors appeared to be involved.

The following procedure might be deduced arbitrarily from the experiments as most likely to give a high percentage of successful preparations. Perfuse with 10% chloral hydrate as recommended by

Hoff. Remove the spinal cord, cut it into blocks 5 to 8 mm. thick, remove the dura, and either put at once into 20% pyridin solution or allow to remain in 10% chloral hydrate for only 1 to 2 hours and then put into the pyridin solution. (If left in chloral hydrate for 24 hours, ammoniated alcohol should be used instead of pyridin.) Two days in pyridin solution should be followed by 2 hours washing with distilled water with changes every half hour. Impregnate with 1.5% silver nitrate solution at 37° C. for 4 days. Wash 20 to 30 minutes using only one water and giving 2 or 3 shakings during the time. Reduce 4 or 5 hours with 2% pyrogallol. After cutting and mounting, sections can be toned with gold by any standard gold-toning procedure. Long reduction in pyrogallol or reduction in formalin-pyrogallol mixtures cause excessive darkening during gold toning.

It should be noted that the method used by Pérez[4] for demonstrating nerve endings in skin is practically identical to that which successfully stains the pericellular endings in spinal cord. His procedure was: 10 or 15% chloral hydrate, 24 hours; rinse; ammoniated alcohol, 24 hours; wash until the blocks just sink in water; 1.5% silver nitrate, 7 to 8 days at 37° C; and reduce with formalin-pyrogallol mixture.

[4]Pérez, R. M. Contribution á l'étude des terminaisons nerveuses dans la peau de la main. Trav. Lab. Biol., Univ. de Madrid. **27**, 187. 1931.

A SIMPLE METHOD FOR CARRYING PROTOZOA IN BULK THRU REAGENTS

E. W. Heil, *Univ. of California, Los Angeles, Calif.*

While working on the neuromotor system of *Paramaecium caudatum* at the University of California, I encountered many exasperating difficulties in carrying these organisms in bulk thru the various reagents of the paraffin embedding and the staining processes. The several methods of conducting the protozoa thru the solutions in boxes, sacks, and tubes of all kinds I found to be awkward and unsatisfactory in many ways. The silk boxes would not keep their shape; it was difficult to strain off the reagents without losing a great percentage of the organisms; or the paramaecia adhered to the sides of the glass tubes and could not be released without serious damage to their structure. Finally, however, I devised the simple method described below.

In histological work there is rather extensively used in the preparation of mesenteric tissue material a certain instrument known as a *histological ring*. More specifically, these rings are called Hoggan's Histological Rings, and are obtainable from Burge, Warren, and Ridgeley, Ltd., 91 Ct., Saffron Hill, London E. C. 1., England. The construction of the ring is as shown in Diagram I, consisting of two parts—a short tube of hard rubber over which is fitted a concentric ring of the same material having a slightly larger diameter. The mesenteric tissue mentioned above is spread over the base of the tube and held stretched out by clamping the ring over it. For my work I merely substituted a square of fine silk bolting (No. 20 quality), as shown in Diagram II. This silk bolting may be secured from any biological supply house in any quality desired. The instrument, as now constructed, can be filled with organisms by simply pipetting into the tube directly from the culture as many as may be desired; thus it is not necessary to centrifuge the protozoa for purposes of concentration. If the culture contains much floating food material, it is advisable first to strain the organisms thru a coarser silk bolting (No. 3 or 4 quality) thereby catching only the organisms themselves in the tube. The protozoa can then be transferred efficiently from one reagent to another, straining thru the silk bolting each preceding solution before placing the organisms into the next one. I found it advisable to put my reagents into shallow Syracuse dishes, because the histological ring will not float in most of the solutions. (See Diagram III). This method also facilitates the microscopic observa-

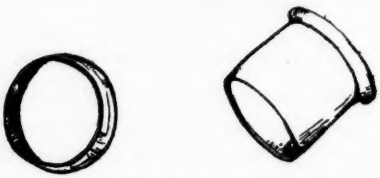

DIAGRAM I

DIAGRAM II

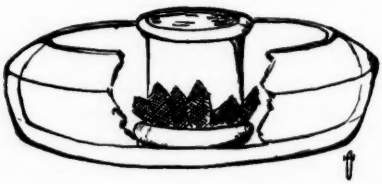

DIAGRAM III

MATERIALS USED IN SIMPLIFIED METHOD FOR CARRYING PROTOZOA IN BULK

tion of the organisms while the process is in operation, for the dish is simply placed on the stage of the microscope and the lens focused down into the tube of the histological ring.

When reagents which prove detrimental to the hard rubber material of the ring are used, the silk bolting may be taken off the tube and set in the Syracuse dish unsupported (the ring will have formed the bolting into a reasonably secure sack after a short period of stretching, especially if the bolting has been previously immersed in alcohol). This step requires more care than do those in which the bolting is secured by the ring, but a little care will insure its safe conduct thru this stage. Xylol and hot paraffin, for example, are quite harmful to the ring material after a time, but if the ring is washed in cold water immediately after the completion of the process, it will last for a reasonably long time.

Very few of the organisms are lost by this method, because the great majority are caught in the mesh of the fine silk bolting, altho a very few are sometimes prone to pass thru "head first" and become lost when the reagents are strained off. The method also reduces the possibility of diluting the reagents, for practically all the preceding solution can be strained off before placing the ring in the next one. Not only is this method applicable to protozoa but to many microscopic and semi-microscopic plant and animal tissues as well. Other qualities of silk bolting may be used for varying sizes of organisms; it is advisable to use the coarsest bolting possible, for the organisms sometimes tend to clog up the mesh if they are much larger than the spaces between the silk strands. It is possible to obtain qualities ranging from No. 0000 (the very coarsest) to No. 21 (an exceedingly fine mesh), which will take care of all but the smaller organisms.

I wish to thank Dr. Gordon H. Ball of the Department of Zoology, University of California, Los Angeles, for his many helpful suggestions and Miss Beatrice Hecht for the drawings.

THE USE OF SALICYLIC ACID AS A FIXATIVE[1]

ISADORE COHEN, *Tufts College, Mass.*

ABSTRACT.—**The following salicylic acid-containing fixatives are useful for cytological studies in plants. The first, here designated HFC, is recommended for studies on somatic mitosis and chromosome individuality. The second, denoted HFP, is recommended for studies on plastids.**

HFC is made up in two solutions. Sol. A: 100 cc. sat. aq. sol. salicylic acid, slight excess copper hydroxide, 20 cc. formaldehyde, 30 cc. normal ortho-phosphoric acid, 200 cc. water, 1 g. saponin; pH 1.8 to 1.9. Fix in Sol. A 15 to 30 minutes in partial vacuum of 35 cms. Then add Sol. B: 1% aq. chromic acid in equal parts. Continue fixation for period of 18 to 24 hours.

HFP is also made up in two solutions which are used in equal parts. Sol. A: 100 cc. sat. aq. sol. salicylic acid, slight excess copper hydroxide, 10 cc. normal ortho-phosphoric acid, ½ g. saponin. Sol. B: 187.5 cc. 1% aq. chromic acid, 50 cc. 2% osmic acid. Fixation technic as HFC.

Dehydrate and infiltrate with paraffin after Zirkle. Stain with crystal-violet-iodine.

The rapid rate of penetration and toxic properties of salicylic acid have apparently never been successfully incorporated in fixing formulae yet published. In the course of the writer's investigation of chromosome behavior in the root tip mitosis of *Narcissus poeticus*, however, two fixing fluids of this nature were developed which have cytological merit. These fluids, one for chromosomes hereby designated HFC, and the other for plastids, denoted HFP, are unique in that they incorporate salicylic acid in their formulae. A search thru the literature reveals only that Heidenhain (1899) used a saturated solution of salicylic acid in one-third alcohol. Lee (1913) in listing this fluid says, "*A trial has given me simply atrocious results.*"

Jacobs (1924) calls attention to the findings of Crozier on the rate of penetration of acids into the mantle cells of *Chromodoris* and of Harvey into the testis cells of *Stichopus* that salicylic acid penetrates much more rapidly than formic, propionic, (Zirkle, 1931) or acetic acid, all of which have been employed in cytological fixing fluids.

[1]The major portion of this work is from a thesis (unpublished) presented by the author at Tufts College, June 1933 for the degree of Master of Science, entitled: Study of root tip mitosis in Hyacinth and Narcissus. I. The effect of various fixatives on chromosome structure.

Since an essential requirement of a good fixing fluid is the rapid penetration of the lethal substance, salicylic acid was experimentally used and the development of the HFC and HFP fluids was the direct result.

Preparation of Fixative HFC

Solution A: Saturate 100 cc. water with salicylic acid by boiling. Salicylic acid is 0.23% soluble at 20° C. While the solution is still hot, add slight excess (0.5 g.) copper (cupric) hydroxide and 30 cc. normal ortho-phosphoric acid. Add 200 cc. water, 20 cc. formaldehyde, and 1 g. saponin. The solution should have a pH of 1.8 to 1.9.

Solution B: Make up 350 cc. 1% aqueous chromic acid.

Solution A might be used alone as a fixative, but the material does not pass thru the processes of dehydration and infiltration satisfactorily. Chromic acid obviates this difficulty.

Fixation with HFC

Immerse material (root tips, ovaries, buds, etc.) in solution A for a period of 15 to 30 minutes and then add an equal amount of solution B. The initial part of fixation should be conducted in a partial vacuum of 35 cms. to facilitate penetration. A fixation period of 18 to 24 hours is sufficient for most objects.

Preparation of Fixative HFP

Solution A: Saturate 100 cc. water with salicylic acid and add excess copper hydroxide as in the preparation of the HFC fluid. While still hot add 10 cc. normal ortho-phosphoric acid and ½ g. saponin.

Solution B: To 187.5 cc. 1% aqu. chromic acid add 1 g. osmic acid and 50 cc. water.

To fix root tips follow the fixation technic given for the HFC fluid. Period of fixation 18 to 24 hours.

Treatment after Fixation

Zirkle's butyl alcohol series is recommended for dehydration and infiltration with 53° C. paraffin (Zirkle, 1930).

A crystal-violet-iodine technic was extensively used for staining. The crystal violet used was a product of the National Aniline & Chemical Co., Certification No. NC-13.

1% aqu. crystal violet (Prepared by boiling 1 g. dye in 100 cc. water for several minutes; cooled and then filtered)... 1 minute
95% alcohol ..... rinse
Iodine solution (1 g. I, 2 g. KI, and 100 cc. 70% alcohol) ..... 15 to 30 seconds
95% alcohol ..... rinse
Clove oil until properly differentiated
Xylol ..... wash
Clear in Xylol ..... 15 minutes
Mount in Canada balsam
If differentiation in clove oil proceeds too rapidly, washing in water after crystal violet is recommended.

The Fixation Image with HFC

In Narcissus root tips, resting nuclei are in some instances fixed with an amoeboid appearance, suggestive of Sakamura's (1920) figures of chloralized nuclei. The mitotic figures are excellently preserved. Prophase nuclei exhibit well-defined, paired chromomeric threads. The individuality of the metaphase chromatids is not obscured by clumping as in other fluids. Spindle and trabant attachments are sharply fixed. The *tassement polaire* stage is so conditioned with this fixative as to permit successful differentiation, thus revealing details which ordinarily are difficult to observe at this stage.

In the root cap and region of elongation, starch grains are preserved and stained.

The HFC fluid was tested and found to be satisfactory with root tips of *Hyacinthus, Allium cepa, Allium sativum, Narcissus poeticus;* ovaries of *Narcissus poeticus;* buds of *Lycopersicum.*

Fixation Image with HFP

Altho resting and dividing chromatin is fixed with this fluid, its chief merit lies in its ability to fix plastids excellently. The formation of starch grains in the root tip of *Narcissus poeticus* can be traced in a single leucoplast in the region of elongation. At times the leucoplast acquires such volume that it pushes the nucleus flat against the cell wall. The disorganization of the leucoplast into starch grains can readily be observed and followed.

The Use of Phosphoric Acid

A consideration of the reagents employed in the HFC fluid would be too lengthy here. With the exception of salicylic and phosphoric acid, the constituents appear in other fixing formulae.

The incorporation of phosphoric acid into the HFC fluid should be explained, as its use in this connection is novel. In the course of de-

veloping this fluid, it was at first thought that the hydrogen-ion concentration was so reduced thru the buffering action of the cell that a basic fixation as described by Zirkle (1928) followed. Phosphoric acid is used to increase the hydrogen-ion concentration of solution A. Its choice is based on its slow rate of penetration and ionizing properties.

The basic fixation may be due to the modification of the primary salicylic acid fixation thru the action of formaldehyde, (Zirkle, 1933). In accordance with this view, phosphoric acid prevents the modifying action of formaldehyde on the primary acid fixation image.

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NOTES ON TECHNIC

PHLOXINE WITH ORANGE G AS A DIFFERENTIAL COUNTERSTAIN.—Phloxine and orange G, following Heidenhain's or Harris' hematoxylin, may be used for differential histologic staining of plant and animal material.

After the sections have been stained in hematoxylin and destained as desired, they are transferred from distilled water to a 0.5% solution of phloxine in 20% alcohol for one or more minutes. They are then rinsed in 70% alcohol, after which a 0.5% solution of orange G in 95% alcohol is dripped upon the sections for 20 to 30 seconds or more. Dehydration in absolute alcohol, clearing in xylol and mounting in balsam follow. Naturally, the exact time of staining must be determined for every kind of material used.

We thus obtained good results with hyaline cartilage in the trachea (guinea pig, Zenker's fixation), and with fetal material (cat's embryo, Ruffini's fixation). After Harris' hematoxylin the matrix stains a light to medium yellow, while the cytoplasm of the cartilage cells is pink with blue or purple nuclei. In cases where the cells completely fill the cavity, the pink cytoplasm lies in justaposition to the yellow matrix, which renders them a very striking appearance. In the dividing cartilage cells, the spindle fibers stand out as pink strands. The chondriogenic layer is pink.

Cilia of the stratified ciliated columnar epithelium of the esophagus stain light orange, epithelial cells pink, mucosa yellow, blood cells and muscle and glandular cells pink.

With plant material the method seems of particular interest for studies of the evolution and disappearance of the spindle. Tradescantia buds, fixed in Ruffini's solution and stained with iron hematoxylin, showed pink spindles, equatorial plates and polar caps in dividing vegetative cells and in the division of the pollen nucleus into the vegetative and generative nuclei. Cyclamen buds (Allen's fixation) gave similar results, but Ruffini's fluid is better suited for the demonstration of the spindle fibers.

Phloxine and orange G, following iron hematoxylin staining, give the following results with floral buds: Chromatin and nucleoli black; cytoplasm pink; spindle fibers usually a pink more intense than that of the cytoplasm; chloroplasts (if sufficiently decolorized) a pinkish orange; cellulose, lignin, cutin are all yellow.—LUBOW A. MARGOLENA, Geneva, N. Y.

A Modification of Schaudinn's Fixative for Protozoa.—One of the most common fixatives for protozoa is Schaudinn's fluid. This is made by adding one part of 95% or absolute alcohol to two parts of saturated aqueous solution of mercuric chloride (6 g. in 100 cc. of distilled water), and is most frequently used with the addition of 1 to 5% glacial acetic acid.

In the past, workers have not always stated whether or not glacial acetic acid was added. If the acid was used, the percentage is often not stated.

In the experience of the writers, the presence of acetic acid and the precise percentage used has a decided effect on the fixation obtained. Evidence for this has been gained by experimental fixations using no glacial acetic acid, 2, 5, 10, 15, and 20% glacial acetic acid. The best fixation varies with the type of protozoan being fixed and the type of structures to be demonstrated. In some cases, the fixative with no acetic acid is the best, whereas as much as 20% is necessary to show some structures.

Along with the above experimentation, good cytological fixation of small flagellates was difficult to obtain on account of the shrinkage and distortion caused by the full strength Schaudinn's fixative. Consequently, a series of dilutions of the fixative have been tried out and as a result the combination of one-half strength Schaudinn's fixative with 2% glacial acetic acid added immediately before using has been adopted. This modification is not only more economical, but the fixation obtained of parasitic protozoa such as *Hexamita*, *Chilomastix*, *Trichomonas*, flagellates of termites, intestinal amoebae and intestinal ciliates has excelled or equalled the fixation with the full strength fixative. This modification is, therefore, recommended to other workers.—D. H. Wenrich and Q. M. Geiman, Zoological Lab., University of Pennsylvania.

LABORATORY HINTS FROM THE LITERATURE

Under this heading are given interesting new methods in microscopy that have been noticed in other publications. The readers' assistance is urged in calling the editor's attention to any outstanding methods in this field that are overlooked.

CLARA, M. Über einige neue einfache gleichzeitige (simultane) Färbungen. Zts. Wis. Mikr., **49**, 348–52. 1932.

Hollborn & Son (Leipzig) offer new stain combinations and a hematoxylin which does not require ripening. No mordanting is necessary and the time of staining ranges from 20 to 40 minutes.

Hematoxylin H is used as follows: 1 g. is dissolved in 100 cc. of hot distilled water, cooled and filtered. This gives a sharp chromatin stain, the cytoplasm remains colorless or shows a grayish or light violet tone. Any of the customary counterstains may be used.

Nucplascoll is a differential stain, the main component of which is hematoxylin H. It is used in a 2% aqueous solution (dissolve in hot distilled water, cool, and filter) on paraffin, celloidin or frozen sections. The sections are transferred to this from distilled water for 30 to 40 minutes, then rinsed, dehydrated and mounted. Nucplascoll gives the following results: nuclei distinct violet-black, greenish black, or brownish black; nucleoli reddish; nuclear membrane clearly outlined; and spindle fibers are stained when present. The cytoplasm is light grayish-green or yellowish-brown (depending upon the fixatives), erythrocytes yellow-red, and collagen fibers green. There is little cytoplasmic differentiation; but for nuclear studies and measurements nucplascoll is preferred to any other iron hematoxylin preparation.

Hematoxylin H also gives excellent results with Van Gieson's Elastin H (Hollborn's picric-acid-fuchsin-orcein mixture). The hematoxylin is allowed to act for 10 to 20 minutes, the sections are then rinsed and transferred to 0.5% Van Gieson's Elastin H in 70% alcohol for 1.5 hours, dehydrated and mounted. Results: nuclei black, elastic fibers brown, connective tissue red, cytoplasm of other tissue yellow. Neither nucplascoll nor hematoxylin H render tissue brittle, as the usual hematoxylin does.

Another differential stain recently offered is Hollborn's "Kernechtrot Kombination H". This is used in the same manner as nucplascoll, except that the sections should be watched as overstaining is undesirable. Results following formalin, sublimate combinations, or alcohol fixation are similar to good Azan staining—nuclei sharp red, collagen fibers bright blue, erythrocytes yellow, cytoplasm varies from a weak bluish to reddish. The "Kernechtrot Kombination H" is preferred to the Azan method because of the ease and quickness of its application. It is similar to nucplascoll, and is an excellent nuclear and connective tissue stain. For finer cytoplasmic structures, however, it has to be supplemented with Azan and the original Mallory stain.

Kernechtrot may also be used as a nuclear stain alone. In this case a 0.1% solution in 5% ammonia alum is recommended for 1 to 5 minutes. Mallory's connective tissue stain may follow Kernechtrot.

GRAY, P. Notes on the practice of fixation for animal tissues. J. Royal Micr. Soc., **53**, 13–19. 1933.

A number of fixatives are recommended according to whether the general shape of whole or large portions of animals, cytoplasmic, or nuclear detail of small objects or portions are to be studied.

The majority of fixatives are unstable and should be freshly prepared before use. Their components, however, form stable solutions—here referred to as "basal fixative solutions"—which may be stored for a considerable period of time. Very few published formulae, however, refer to preparation from solutions, and even where this is the case, the solutions quoted are not constant thruout the whole series. It has been found possible, by dividing all the fixatives quoted into an "alcoholic" and "aqueous" series, to devise 10 aqueous and 2 alcoholic "basal fixative solutions" which, when mixed with each other and with ordinary bench reagents, will give the whole range of formulae quoted; they can also, by a simple mathematical adjustment, be made to give any other fixative which is likely to be required.

There is included a table giving various combinations of these basal fixatives, enabling one to prepare 55 standard fixing fluids.

HANCE, R. T. A new paraffin embedding mixture. Science, **77**, 353. 1933.

The following embedding mixture is recommended for good sectioning of biological material: A stock solution of crude rubber in paraffin is first made. Crude rubber is available in thin sheets, either smoked or unsmoked. Both kinds work equally well. The sheets of crude rubber are chopped up with scissors and dropped in melted Parawax or any similar common paraffin. The paraffin should be smoking hot and the mixture should be stirred occasionally. Three to four hours are required to completely melt the rubber. About 20 g. of rubber can be dissolved in 100 g. of paraffin.

Embedding mixture. Paraffin 100 g., rubber-paraffin mixture 4 to 5 g., beeswax 1 g. Filter. The mixture is pale yellow in color, does not crystallize readily, and is of a waxy consistency that sections unusually well.

It has been used at the University of Pittsburgh for 3 years and has materially increased the success of large classes in micrology.

HRUBY, K. Double staining by the Cajal-Brozek method. Science, **77**, 352–353. 1933.

Professor A. Brozek has introduced and modified Ramon y Cajal's staining so as to apply it to botanical technic. The procedure has been successfully used for 3 years for cytologic work at the Charles University at Prague. Fixation has to be sufficiently acid in order to obtain good results. Nawashin's formula (15 pts. 1% chromic acid, 4 pts. formol, 1.5 pts. glacial acetic acid) is recommended above all others. This solution has been tried with success even after fixing in highly diluted HNO_3. Nemec's fixative I is not recommended, but II may be used (to wit, 50 cc. of 1% chromic acid, 50 cc. of 3% bichromate of potash, 8 cc. of formol); Regaud's fixative may be used (80 pts. 3% bichromate of potash and 20 pts. of formol); also sublimate with acetic acid.

Stain paraffin-freed sections in saturated aqueous magenta (basic fuchsin) for 3 to 5 minutes, and rinse in distilled water. Transfer to picro-indigo-carmin, (2 pts. of saturated aqueous indigo carmin and 1 pt. of picric acid) for 10 to 15 minutes. (Source and dye content of stains not given). Time should be varied to suit different material. Rinse in slightly acidulated water for 1 minute. Differentiate in 80% alcohol for 30 to 60 seconds, until the red ceases to come out of the sections. Dehydrate, clear, and mount.

The chromosomes should be bright red, nucleoli and other parts sky blue. The procedure is time-saving (40 to 45 minutes only) and is much preferred to iron hematoxylin because the chromosomes stained with fuchsin can be well distinguished from one another, even when they lie close to each other, or actually on each other. Slides thus obtained can be studied in all details, drawn and excellently photographed with a green filter.

MCNAMARA, W. L. Giemsa stain for tissue, rapid method. J. Lab. & Clin. Med., **18**, 752, 1933.

The usual procedure of staining sections with the Giemsa stain consumes 8 to 12 hours. The following rapid modification is in use at the Edward Hines, Jr., Hospital, and the results are entirely satisfactory.

Fix in Zenker's fluid for 48 hours or more. Wash for 24 hours. Bring up to paraffin and section 4 to 6 μ thick. Run down to water. Treat with Lugol's solution (5 cc. to 100 cc. of distilled water) for 30 min. followed by one change of 95% alcohol and one of tap water. Treat for 10 min. in a 0.5% solution of sodium hyposulfite and wash well in tap water.

Stain for 15 min. in the following: Giemsa stain (stock sol.), 10 cc; acetone, C.P., 10 cc; methyl alcohol, C.P., 10 cc; 0.5% sodium carbonate, 2 M; distilled water, 100 cc. Wash rapidly in water and differentiate in S. B. Wolbach's mixture (colophonium 15 gm; acetone 100 cc). Treat the sections separately, changing the solution once. Renew as soon as the precipitate of colophonium fails to dissolve quickly. Differentiation usually takes 15 to 45 seconds. Pass thru a mixture of 7 parts acetone and 3 parts xylol. Follow with xylol and mount in cedarwood oil.

WINTREBERT, P. La conservation dans les coupes de paraffin des marques vitales au brun Bismarck et au sulfate de bleu de Nil, faites sur les oeufs vivants d'Amphibiens. Compt. Rend. Acad. Sci. Paris, **194**, 1013–15. 1932. (From abstract in Zts. Wis. Mikr., **49**, 486, 1933).

Vitally stained (Bismarck brown and Nile blue sulfate) amphibian eggs may be preserved by the following procedure: Fix 1 hour in Zenker's solution without acetic acid, saturated with picric acid, or preferably with 5% sublimate containing 2 parts of phosphomolybdic acid, and then picric acid added to saturation. Transfer for at least 24 hours to a saturated solution of picric acid containing 1.5 parts phosphomolybdic acid to 100 parts of the solution. Dehydrate in 15, 30, 50, and 80% alcohol saturated with picric acid and containing 1:100 parts of phosphomolybdic acid, leaving the objects 3 minutes in each, then in a mixture of equal parts of 80% alcohol and terpineol. Transfer thru toluene to paraffin. Sections may be counterstained with hemalum. Vitally stained cells show up well on the yellow background.

ZIRKLE, C. Vacuoles in primary meristems. Zts. Zellf. & Mikr. Anat., Heft 1, 26–47. 1932.

Vacuoles in the primary meristems of *Pinus, Polygonum, Robinia, Phaseolus, Fraxinus, Zea, Osmunda,* and *Lunularia* were investigated in both living and fixed preparations. The usual acid fixatives completely destroy the true vacuoles and form artificial ones. Fixatives which contain acetic acid, any acetate, chromic acid, or a bichromate on the acid side of pH 4.2, give an entirely erroneous picture of the cytoplasm and vacuoles.

Vacuoles containing tanin may be blackened by the Golgi technic. They can also be fixed and stained by mixtures of formaldehyde and iron salts. Modifications of mitochondrial fixatives preserve the true form of the cytoplasm and vacuoles, in spite of the fact that the vacuolar membrane is destroyed. When the vacuoles contain no tanin, they appear as colorless regions clearly outlined by the faintly stained hyaloplasm.

The following mixture penetrates well and preserves the size and shape of the vacuoles: $K_2Cr_2O_7$, 1.25 g.; $(NH_4)_2Cr_2O_7$, 0.25 g.; $CuSO_4$, 1 g.; formalin (40%), 10 cc.; water, 90 cc., the formalin added immediately before fixation, which should be completed by 48 hours. This unstable fixing fluid may be replaced by a stable one: $Cr_2(SO_4)_3 \cdot 15\ H_2O$, 5 g.; CuO, slight excess; formalin, 100 cc. In this formula the best concentration of formalin varies from 1:9 to 1:1, depending upon the material, and must be determined empirically. Too great a concentration causes some plasmolysis and a swelling of the vacuoles; if too little is used it is so diluted by the contents of the tissue that the mixture gives an erratic fixation image.

ZIRKLE, C. Cytological fixation with the lower fatty acids, their compounds, and derivatives. Protoplasma, **18**, 90–111. 1933.

The cytological fixation images (plant material) of the following 4 interlocking series of organic acids, together with their copper and nickel salts, were investigated: A) formic, acetic, propionic, butyric, valeric; B) acetic, tricholroacetic; C) formic, glycolic, glyceric, gluconic; D) glyceric, lactic, propionic.

Each acid and salt was mixed with formaldehyde and the resulting fixation images recorded. Copper bichromate was mixed with each copper salt with which it was stable and specimens fixed in each mixture.

Acids used alone cause the cytoplasm to shrink. Fixation by copper salts were like those of the acids, with a lesser shrinkage, and mordanted nucleoli. Nickel salts did not fix. The action of acids on formalin fixation is discussed. The rate of penetration and action of the various components of compound fixatives is described.

Several acid images obtained show plastin in such a way that it can be followed thru cell division. The mordanting of both plastin and chromatin is determined primarily by the H-ion concentration at which fixation occurs. A strong acid (formic), plus formaldehyde, mordants the chromatin but not the plastin. The copper salts of the fatty acids, plus either formaldehyde or copper bichromate at pH 4.5–5.0, fix and mordant both plastin and chromatin. The nickel salts of the fatty acids, plus nickel bichromate, fix at pH 5.2–5.4 and mordant the plastin, but not the chromatin.

Stains Recently Certified

In the table below is given a list of the batches of stain approved since the last one listed in the July number of this journal.

Stains Certified June 1, 1933, to August 31, 1933*

Name of dye	Certification No. of batch	Dye Content	Objects of tests made by Commission†	Date approved
Basic fuchsin	CF 14	91%	For use as bacteriological stain, and in Endo medium	June 13, 1933
Basic fuchsin	NF 23	77%	For use as bacteriological stain, and in Endo medium	June 19, 1933
Crystal violet	NC 14	90%	For use as histological and cytological stain	July 14, 1933
Congo red	LQ 4	70%	For histological use	July 31, 1933

*The name of the company submitting any one of these dyes will be furnished on request.

†It is not to be inferred that these are the only uses for which each of these samples may be employed. The Commission ordinarily tests each dye for such of its comman uses as seem to give the most severe check as to its staining value. Certification does not in any instance, however, imply approval for medicinal use.

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